# Stimulation of taxol production by combined salicylic acid elicitation and sonication in *Taxus baccata* cell culture

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*Abstract*-**This study surveys the effects of salicylic acid (SA) and ultrasound (US), as a physical stimulus and their combined use on the growth and taxol production of *Taxus baccata* cells in suspension culture. The cultures were treated with SA at concentrations of 25 and 50 mg/L, low frequency US (40 KHz) for a short period of time (2 min) and their combined use. SA at high concentration and in combined treatment significantly decreased growth and viability of the cells but the treatment of cells by US individually didn't have any significant effect on the parameters. The hydrogen peroxide content and level of lipid peroxidation were increased by all treatments in compared to control and in this respect, US enhanced effect of SA. Phenolics content and taxol biosynthesis of the cells were significantly increased under effect of treatments. Increase in SA concentration resulted in more taxol production and most yield of taxol was obtained at 50mg/L of SA which enhanced about 6.6-fold in compared to control. SA at all levels was more effective than US in stimulating cell-associated and total taxol production. The extracellular taxol was more affected by US exposure which was about 3 times higher than that of the control. Combined use of US and SA at 50 mg/L caused most improvement in total taxol production, which was about 4, 1.2 and 8 times higher than that of the US, SA, and control, respectively. Through the results it is suggested that US can act as an abiotic elicitor to induce secondary metabolite production such as taxol in *Taxus* cell cultures.**

*Keywords*-*Taxus baccata, taxol, cell culture, ultrasound, salicylic acid*

## I. INTRODUCTION

Taxol, a highly effective anticancer drug with unique chemical structure, is produced by limited organisms such as yew plant*,* hazelnut, and some microorganisms [1, 2]. The natural supply of the drug from the plant is only about 0.01%~0.06% [3] and necessitates the investigation for alternative sources. Plant cell culture has been recognized as a potential alternative of producing taxol in a large scale [4]. At present, a lot of methods have been developed to overcome this problem. Elicitation is one of the efficient methods which stimulates the pathways of defense-related secondary metabolites [5]. It has been shown that SA plays an important signaling role in activation of various plant defense responses and SA-elicited plant cell cultures resulted in a dramatic increase in production of secondary metabolites such as taxol [6, 7].

US is a physical or mechanical stimulus that various biological effects has attributed to it [8]. Despite the high-intensity US, which is generally destructive to biological materials, increasing interest has been paid to the low-intensity or mild US and its potential applications in biological systems and biotechnological processes [9]. Mild US may stimulate biological activities, such as secondary metabolite production [10] plant defense responses, transmembrane influxes, and oxidative burst in plant cell cultures [11] as well as cellular transporters activity [9].

A synergistic effect of multiple elicitors on production of secondary metabolites in plant or cell cultures is often observed [5]. SA, a proven signal compound in the elicitation of plant defense responses, stimulates secondary metabolite production in plant cell cultures. Low-energy ultrasound can act as an abiotic elicitor [11] and can induce cell membrane permeabilization to enhance intracellular product release. To the best of our knowledge, the interaction between the two chemical and physical elicitors on plant cell culture has not been investigated. Therefore, in this work, suspension cultures of *Taxus baccata* were exposed to them and taxol production and other biological changes were then assayed.

## II. MATERIAL AND METHOD

### A. Establishment of cell culture and elicitors treatment

Calli were induced from longitudinally-halved stem sections of *T. baccata* L. on solidified B5 medium, supplemented with NAA (2 mg/l), 2,4-D (0.2 mg/l), BA (0.2 mg/l), sucrose (20 g/l) ascorbic acid (50 mg/l) and pH 5.5 for about 20-30 days. Cell suspensions were established from the friable calli in the same media without agar. The cultures were incubated at 25°C in the darkness, on orbital shakers (120 rpm) and were subcultured every 2 weeks. Ethanolic solution of SA (70%, v/v) was sterilized by filtration (0.2μm) and added to media at final concentrations of 25 and 50 mg/L on day 8 of subculture. For US exposure, the cultures were sonicated with low frequency US (40 KHz), 2 min on day 16 in the ultrasonic bath (FALC Instruments, Italy). In combined tests, a group of cells in suspension culture were treated with 25 or 50 mg/l SA on day 8 and exposed to US 2 min on day 16. Samples for

control and supplemented cultures were harvested on day 24 and frozen in liquid $N_2$ and kept at -80 $^0$C until used for biochemical analysis.

### B. *Determination of growth and viability and measurement of biochemical parameters*

Cell growth was evaluated by measuring the increase of cell dry weight. Viability assay was performed with Evans Blue [12]. Hydrogen peroxide content was assayed according to the method as described [13]. Level of damage of membranes was determined by measuring malonyl dialdehyde (MDA) as the end product of peroxidation of membrane lipids [14]. The phenolic content of cells was determined by Folin–Ciocalteu method [15], using gallic acid as a standard.

### C. *Taxol extraction and analysis*

Taxol was extracted from medium and cells by methods as previously described [11]. The taxol content was analyzed by HPLC system (Knauer, Germany), which was equipped a C-18 column (Perfectsil Target ODS-3 (5μm), 250 × 4.6 mm) MZ-Analysentechnik, Mainz, Germany). Taxol was eluted at a flow rate of 1 ml methanol and water (45:55, v/v) min$^{-1}$ and was detected at 227 nm using a UV detector (PDA, Germany). Identification of taxol was accomplished by comparison of retention times with authentic standard (Sigma).

### D. *Statistics*

All experiments were repeated at least three times with similar results. The data shown in the figures and table are mean values ± S.D.

### III.    RESULTS AND DISCUSSION

#### A. *Effects of SA and US on biomass production and viability*

Figure 1 shows the effect of SA and US exposure on the growth (dry matter) and viability of *Taxus baccata* cells. Increase in SA concentration, resulted in significant decrease in the growth and viability of cells compared to control cultures. The results are in agreement with those found by [16] on the effects of SA on *Salvia miltiorrhiza* cultured cell, and imply that response to SA is dose dependent. In addition it has been reported that the cell death of *Taxus cuspidata* cell cultures was significantly induced by SA [17]. Exposure to US did not significantly change the dry matter and viability but when the cells were pretreated with SA and then were exposed to US, the two measured parameters were lower than those of the control cells or those which were treated with either US or SA. This indicates a synergism between SA and US. Similarly, short US exposure did not lead to significant depression of the biomass production in *Taxus chinensis* cell cultures [11]. The energy given off by US affects the cell and causes disruptions and damages in the plasma membrane, which somewhat may slow down normal cell growth. The cells try

to repair the damage with time, as shown by [18]. In SA-pretreated cells however, the scenario may completely be changed and the growth and viability could not be restored. It has been shown that SA inhibits mitochondrial electron
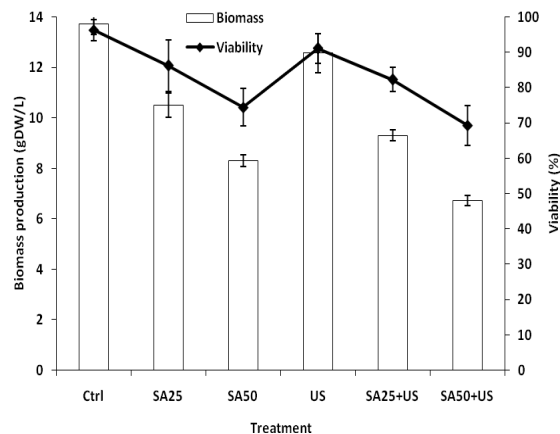


Figure 1. Effect of SA and US on biomass and viability of cell suspension cultures of *Taxus baccata*. (SA and US were treated on days 8 and 16 post inoculation, respectively). DW= dry weight. Data are mean ± SD, n = 3.

transport leading to ATP exhaustion [19]. Combination of low ATP levels with ROS such as $H_2O_2$ which induced by SA (Fig. 3), may resulted in $Ca^{2+}$ influx and cell death [20].

#### B. *Effects of SA and US on $H_2O_2$ production and lipid peroxidation rate*

The effect of SA and US on $H_2O_2$ production and MDA content of ells in *Taxus* suspension cultures has shown in Fig. 2. There was a significant increase in $H_2O_2$ production and MDA content under all treatments compared to that of the control culture. Production of $H_2O_2$ by SA-treated cells increased along with the increase of concentration of SA. Production of $H_2O_2$ was also significantly marked when both elicitors were joined together. Over production of ROS such as $H_2O_2$ is an important plant response to a wide range of biotic and abiotic stresses and also acts as a second messenger to signal subsequent defense reactions [5]. In comparison, production of $H_2O_2$ by US-treated cells was practically similar to that of SA-treated cells. Since some physical distortion of the cell wall/plasma membrane such as degradation, puncture, or deformation may occur during collision of US waves to cell surfaces, a mechanical trigger/signal could explain why US stimulates ROS production or defense responses in *Taxus* cells. Our results are in agreement with those found by [21] that production of $H_2O_2$ was induced in sonicated ginseng cell cultures within 2 min of US exposure. In addition, it has been showed that, the concentration of $H_2O_2$ increased after SA treatment and this was followed by an increase in secondary metabolite artemisinin concentration [22].
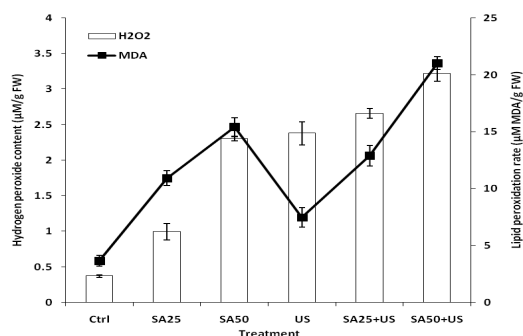
Figure 2. Effect of SA and US on hydrogen peroxide content and lipid peroxidation rate of cell suspension cultures of *Taxus baccata*. (SA and US were treated on days 8 and 16 post inoculation, respectively). FW= Fresh weight. Data are mean ± SD, n = 3.
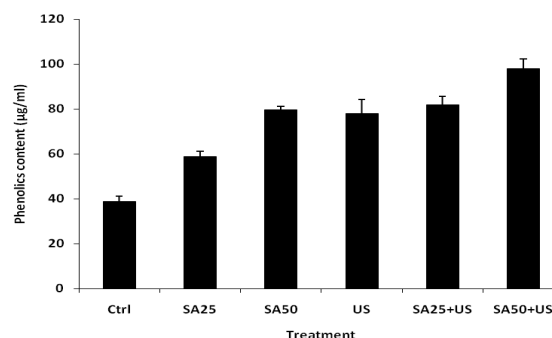


Figure 3. Effect of SA and US on phenolics content of cell suspension cultures of *Taxus baccata*. (SA and US were treated on days 8 and 16 post inoculation, respectively). Data are mean ± SD, n = 3.

There was a direct correlation between SA concentrations and MDA content of cells. Combination of US and SA induced a higher level of MDA content compared to each treatment alone. It has been reported that exogenous SA treatment improved lipid peroxidation rate in plant cell cultures [23]. In addition, MDA content was significantly higher in US-treated microalga *Porphyridium cruentum* compared to control [24]. Comparing the figures 1 and 2 shows that more the level of MDA, more the decrease in biomass and viability. By generating changes in unsaturated fatty acids that affect membrane structure and properties, this enhanced free radical formation and lipid peroxidation under the treatments may have also brought about an increase in membrane permeability or loss of membrane integrity, as evidenced by the increase in extracellular taxol (Table1). The occurrence of lipid peroxidation indicates that cell death is not impaired even in the presence of lipid antioxidants [25]. The $H_2O_2$ production induced by SA and US in *Taxus* cells show that defense responses have stimulated in treated cells, which resulted in elevation of MDA content, cell death, and finally production of phenolics and taxol as defense-related compounds.

*C. Effects of SA and US on phenolics content*

The effect of SA, US and combined use of them on the accumulation of the phenolics content in the treated *Taxus* cell cultures are shown in Fig. 3. After adding SA to each cultured medium, the production of phenolics were dramatically increased compared to that of the control. Increased SA concentration from 25 to 50 mg/L resulted in phenolics accumulation about 1.5 and 2-fold higher than that of untreated cells respectively. Thus the elicitation of *Taxus* cell culture with SA was dependent on the SA dosage. Interestingly, upon treatment with US, phenolics content was significantly higher than that of control and also the same as of SA treated cells (Fig. 5). US exposure strengthened the SA effect on phenolics production in combined treatments and this was dependent on SA concentration, indicating a synergistic effect of SA and US. The present work has shown that SA elicitation resulted in

a decrease in the biomass production (Fig. 1) while increasing the phenolic compounds accumulation (Fig. 3). Similar trends have been reported for cell cultures of *Salvia miltiorrhiza* [16] and *Hypericum perforatum* [26] after elicitation with SA.

The improvement of phenolics accumulation in *Taxus* cell culture by low-intensity US implies that the biosynthesis of the compounds may be stimulated by mechanical triggers. Since phenolics are the metabolic products in phenylpropanoid pathway as a result of the response of plant cells to environmental stress [27], the increase in phenolics accumulation indicates that the secondary metabolisms of cells were enhanced. A few previous works have shown increase in secondary metabolites production in plant cells after treatment with US, saponins in *Panax ginseng* cells [21], shikonins in *Lithospermum erythrorhizon* cells [10], and taxol in *Taxus chinensis* cell cultures [11]. The results obtained here may point to that eliciting *Taxus* cell by SA or US down regulated primary metabolism in benefit to secondary metabolism. Considering the inverse link between the growth and the accumulation of secondary metabolites, the cell growth inhibition elicited by SA, US and their combination treatments may augment the production of secondary metabolites.

*D. Effects of SA and US on taxol production*

The effects of the treatments on production of taxol in *Taxus* suspension cultures are shown in Table 1. All treatments led to significant increase in the extracellular, cell-associated and total taxol accumulation compared to control. The increase in SA dose led to significant increase in the both extracellular and cell-associated taxol accumulation compared to control. SA was more effective in stimulating extracellular taxol production and the highest amount for extracellular taxol was achieved at 50 mg/L of SA, 19.44 mg/L, which were about 12.6-fold that of the control culture. In fact, the increase in the amount of extracellular taxol accounted for most of the improvements of total taxol yield obtained with the SA levels. SA increased the total taxol production significantly about 4-

fold at 25 mg/L and 6.6-fold at 50 mg/L that of the control culture.

The treatment of cells with US had significant effect on taxol production. The extracellular taxol was more affected by US application to cell suspension cultures compared to cell-associated taxol. This means that the release of taxol stimulated the further synthesis of taxol in the cell. On average, the US exposure increased the extracellular, cell-associated and total taxol yield 3-, 1.2- and 2-fold compared to the control, respectively. SA at all levels was more effective than US in stimulating taxol production. The exposure of US to cultures treated with SA led to more increase in the taxol production (Table 1). In this respect the effect of US on taxol production was significantly dependent on the SA concentration. Especially, combined treatment of US and SA at 50 mg/L caused significant improvement in total taxol production. This was about 4 times higher than that of the culture treated with US, 1.2 times higher than that of the culture elicited with SA at 50 mg/L, and 8 times higher than that of the control. In addition, the exposure of US significantly increased extracellular taxol in SA-treated cultures compared to those elicited with SA and US alone.

The results obtained here showed that combined use of US and SA at concentration of 50 mg/L resulted in maximum accumulation of taxol and phenolics which was paralleled by a significant increase in hydrogen peroxide induction and lipid peroxidation rate, and other hand significant decrease in cell growth and viability. These results are in agreement with those found by [28] which, concluded that cell death was closely related to taxol production in fungal elicited cell suspension cultures of *Taxus chinensis*. Our results showed that US exposure potentiated the SA effect on taxol and phenolics production and it was dependent to SA concentration (Fig. 3 and Table 1). There are a few researches evidenced that elicitor mixtures usage often could result in higher taxol production in *Taxus* cell culture. Combined use of elicitors (biotic and abiotic elicitors) in cell suspension cultures of *Taxus chinensis* enhanced taxol production about 40-fold higher

compared to that of the control [29]. Furthermore, it has been shown that the elicitors strengthen each other effects on other secondary metabolites when applied simultaneously. For example, exogenous SA could not induce cryptotanshinone formation in cell culture of *Salvia miltiorrhiza* when applied alone. However, when applied in combination with yeast elicitor, SA had a significant synergic effect on cryptotanshinone formation [30].

The all treatments exposed to cell cultures improved taxol release to the medium (Table 1). The increase in SA level led to significant enhance in the amount of taxol release and the elicitor at 50 mg/L level was more efficient in releasing taxol compared to lower level and control. In agreement with the results obtained here, no alkaloid release was observed from *Atropa belladonna* transformed roots when SA was added at a concentration of 0.5 mM or lower, while after the addition of SA at concentrations higher than 0.5 mM the ratio of alkaloid release increased with increasing SA concentration up to 2 mM [31].

Increase in taxol release of about 62% was observed in cell cultures which were exposed to US. Taxol release in cultures treated by combined use of SA and US was more than that of US and SA alone. The specific yield was also improved by all treatments and the increase in SA level led to significant enhance in the amount of it. US augmented effect of SA in respect to specific yield significantly, suggesting a synergistic accumulative effect (Table 1). Most increase in taxol release and specific yield was observed in cell cultures which were treated by combined use of SA at 50 mg/L and US.

The increased cell permeability by US or SA might be one of the mechanisms, which led to the release of the induced taxol and kept the intracellular taxol at a relatively low level, which in turn favored taxol biosynthesis as well. These findings reveal that utilize SA in a culture system alone and with US exposure to stimulate the production and release of taxol is of practical significance for the biotechnological production of plant secondary metabolites such as taxol if the amount of SA and the exposure of US are appropriately determined.

TABLE I.    EFFECT OF SA AND US ON PHENOLICS CONTENT OF CELL SUSPENSION CULTURES OF *TAXUS BACCATA*.

| SA (mg/L) | US | Taxol (mg/L) | | | Release (%) | Specific yield mg/g cell |
|---|---|---|---|---|---|---|
| | | Extracellular | Cell-associated | Total | | |
| 0 | - | 1.54±0.23 | 2.52±0.65 | 4.06±0.88 | 37.93 | 0.29 |
| | US | 5.17±0.89 | 3.15±0.59 | 8.32±1.48 | 62.13 | 0.62 |
| 25 | - | 10.24±1.56 | 6.65±1.04 | 16.89±2.60 | 60.62 | 1.60 |
| | US | 21.44±3.28 | 5.34±0.82 | 26.78±4.10 | 80.05 | 2.87 |
| 50 | - | 19.44±2.45 | 7.54±0.69 | 26.98±3.14 | 72.05 | 3.25 |
| | US | 28.76±1.78 | 4.24±0.43 | 32.9±2.21 | 87.11 | 4.88 |

SA and US were treated on days 8 and 16 post inoculation, respectively. Data are mean ± SD, n = 3.

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